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Apoptosis in Breast Cancer and Its Relationship to Clinicopathological Characteristics and Prognosis

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Background and Objectives: Apoptosis is essential to maintain homeostasis in living organisms and occurs in a variety of tissues in response to both physiological and pathological stimuli. In breast cancer, most cytotoxic drugs and hormonal treatments induce apoptosis. We studied the relationships between apoptosis and clinicopathological variables or prognosis in 143 patients with operable breast cancer.

Methods: Apoptosis was numerically graded in 5 consecutive 40× high-power fields (HPF) of hematoxylin-eosin (H&E) stained sections, since we showed that there was a significant correlation of H&E staining with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining.

Results: The average number of apoptotic cells was 19.9 (0~168)/5 HPF, and cases were classified into 3 groups based on the number of apoptotic cells/5HPF: 0 to 10, 11 to 30, and 31+. The level of apoptosis increased with increasing size of the tumor, and apoptosis was rarely seen in tumors with positive ER or lower proliferative activity, as assessed by DNA polymerase α. As shown by DNA content analysis, apoptotic cells were observed more frequently in tumors with low G1 and high S-phase fractions. In addition, apoptosis was correlated with overexpression of p53 and poor prognosis. Although apoptosis did not correlate with EIC (extensive intraductal component) status, tumors with comedo component had higher values of apoptosis than those without comedo component.

Conclusions: In breast cancer, apoptosis might reflect biological behavior, namely a higher degree of biological aggressiveness and unfavorable prognosis. *J. Surg. Oncol. 1999;71:226–234.* © 1999 Wiley-Liss, Inc.

KEY WORDS: **breast neoplasms; histological evaluation of apoptotic cells; proliferative activity; DNA analysis; prognostic factors; survival**

INTRODUCTION

Apoptosis is a normal physiological process that maintains homeostasis in living organisms and occurs spontaneously in untreated malignant neoplasms, and is additionally induced in response to both physiological and pathological stimuli. Apoptosis is an active process that depends on the expression of a specific set of genes. Among them, wild-type p53 can induce apoptosis [1,2], whereas mutant p53 can inhibit apoptosis [3–5]. Apoptosis is now thought to be involved in the early stages of cancer formation. In breast cancer, anti-apoptosis bcl-2 protein is frequently expressed, and is correlated with estrogen receptors and favorable prognosis [6–8]. Regarding treatment for breast cancer, most cytotoxic drugs

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induce apoptosis [9,10]and it has been demonstrated [11–13] that antiestrogens or withdrawal of sex steroids increases apoptosis.

In this study, to clarify the role of apoptosis in breast cancer, we examined the relationship between apoptosis and clinicopathological factors and prognosis in primary breast cancer.

MATERIALS AND METHODS

Patients

One hundred and forty-three patients with primary breast cancer who were operated on between November 1989 and July 1994 in Kumamoto City Hospital were studied. The mean age of the patients was 52.2 years (range:30–89). Seventy-six patients were postmenopausal. The average tumor size was 1.5 cm (range:0.5–3.2cm) and 38 cases were lymph node positive. Among them, 28 patients developed recurrences. The most common sites of metastasis were soft tissue (including skin metastasis, subcutaneous tumor, or lymph node metastasis):10 patients; bone: 4 patients; and visceral metastases: 14 patients.

Histological Method

The operative specimens from primary tumors were fixed in buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin. The examined items were histological type, lymphatic invasion (ly), infiltration to fatty tissue (f), extensive intraductal component (EIC), comedo status, and lymph node metastasis. EIC was judged positive when the intraductal cancer component covered 25% or more of the main tumor [14]. The identification of apoptotic cells was carried out by immunostaining with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) method, which incorporates a nucleotide analog onto the free ends of DNA, using antibody (ApopDETEK Cell Death Assay System by Enzo Diagnostic, Inc., New York, NY) and paraffin-embedded sections. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. As positive controls, we used slides from a formalin-fixed, paraffin-embedded mammary carcinoma known to show many apoptotic cells in H&E stained sections. As negative controls, we processed equivalent specimens in parallel substituting phosphate buffered saline (PBS) for the labeling reagent. Apoptotic cells were counted in 40×high-power fields (HPFs) and the cell number was averaged in 5 consecutive HPFs. In hematoxylin-eosin (HE) stained sections, criteria for the identification of apoptosis included the presence of pyknosis and karyorrhexis as characterized by cell shrinkage, nuclear condensation, fragmentation, apoptotic body, and the lack of inflammatory components [15,16]. The number of apoptotic cells was evaluated as in the TUNEL method. P53 proteins were immunohistochemically examined by using mouse monoclonal anti-p53 antibody (Japan Tanner Corporation, Tokyo, Japan), and cases were thereby divided into 3 groups: (−), (+), or (++). A microwave antigen retrieval method was used and the streptavidin-biotin system was applied for p53 . DNA polymerase α immunoreactivity was evaluated using a peroxidase-antiperoxidase (PAP) kit, employing a mouse IgG monoclonal antibody, CL22-4-42B (Medical Biological Laboratory, Tokyo, Japan) [17], in 123 cases. The proliferative activity of each tumor was classified semiquantitatively into one of three groups according to the percentage of positive nuclei: 0 to 19%, 20 to 49%, and 50%+.

Cellular DNA content was analyzed in 114 cases using the method described by Shutte et al. [18]. A suspension of isolated nuclei was prepared from paraffin-embedded sections after confirming the amounts of cancer tissues. The propidium iodide-stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, CA). DNA aneuploid tumors were defined as having more than one G0/1 peak. Cases with only one G0/1 peak were classified as DNA diploid. Analyses of DNA histograms were performed in 106 cases using the method of Dean and Jett [19], and the proportions of cells in G1, S, and G2+M-phase fraction were calculated. These cases were divided into 3 groups according to the proportion of cells in the SPF (S-phase fraction): 0 to 7.9%, 8 to 15.9%, and 16%+, which reflected clinical significance as previously reported [20].

ER was assayed in samples of the primary tumor by the EIA (enzyme immunoassay) method and was considered positive if $\geq$14 fmol/mg protein.

Statistical Methods

The inter-group differences in Tables I and II were examined by Student's paired *t*-test and Analysis of Variance (ANOVA). Multivariate analysis was performed for the values in Table I using Multivariate ANOVA (SPSS). The χ^2 test was employed to test for associations between clinicopathological variables and apoptosis in Tables I and II, and cumulative survival rate after recurrence was calculated by the Kaplan-Meier method and examined by logrank test (median follow-up period: 55 months). To evaluate a risk factor for survival, Cox proportional hazard model was used for uni- and multivariate analyses of cases (SPSS). The relationship between the evaluation of apoptosis by H&E staining and TUNEL staining was evaluated using the Spearman rank correlation coefficient (SPSS).

TABLE I. Apoptosis and Clinicopathological Variables in Breast Cancer*

Variables	No. of cases	Apoptotic cells (mean ± standard deviation)	*P* value (univariate)	*P* value (multivariate)
Age (years)				
−35	4	17 ± 14.0		
36–50	70	17.4 ± 20.0	0.401	
51+	69	22.6 ± 32.8		
Menopausal status				
Pre	76	17.4 ± 19.6	0.23	
Post	67	22.8 ± 33.2		
Tumor size (mm)				
−10	30	10.2 ± 8.3		
11–20	87	19.8 ± 28.6	0.011	0.05
21+	26	31.5 ± 30.6		
Nodal status				
−	105	18.1 ± 23.6	0.18	
+	38	24.9 ± 34.1		
Histological type				
Non-invasive carcinoma	5	7.6 ± 6.7		
Papillotubular carcinoma	22	12.6 ± 14.4	0.004	
Solid-tubular carcinoma	39	34.0 ± 34.0		0.71
Scirrhous carcinoma	66	16.5 ± 19.5	0.014	
Lobular carcinoma	3	10.3 ± 1.5		
Mucinous carcinoma	4	4.3 ± 3.3		
Others	4	17.8 ± 17.7		
ly				
−	61	13.8 ± 16.3		
+	56	23.6 ± 32.4	0.053	0.56
++	26	26.0 ± 31.3		
f				
−	44	18.2 ± 20.5		
+	68	20.0 ± 30.5	0.83	
++	31	22.1 ± 26.9		
EIC				
−	100	21.8 ± 29.9		
Non-comedo	29	10.4 ± 9.3	0.087	0.60
Comedo	14	26.2 ± 24.8		
p53				
−	68	10.8 ± 12.2		
+	41	13.4 ± 14.1	<0.0001	0.007
++	34	46.0 ± 40.4		
DNA polymerase α				
−19 (96)	72	11.3 ± 10.6		
20–49	31	36.0 ± 36.1	<0.0001	0.03
50+	20	37.0 ± 41.2		
Unknown	20	9.0 ± 8.9		
ER				
+	78	12.6 ± 14.6	0.0002	0.94
−	50	34.9 ± 37.2		
Unknown	15	8.0 ± 7.7		

*ly, lymphatic invasion; f, infiltration to fatty tissue; EIC, extensive intraductal component; ER, estrogen receptor.

RESULTS

Comparison Between Apoptosis Evaluated by TUNEL Staining and H&E Staining

Figure 1a shows apoptosis as revealed by TUNEL staining, and Figure 1b shows apoptosis as revealed by H&E staining. The correlation of measured values of both stains in 5 consecutive HPFs in 20 cases involved in this study are shown in Figure 1; there was a significant correlation between them ($P < 0.001$). Accordingly, the evaluation of apoptosis by H&E staining was adopted in this study.

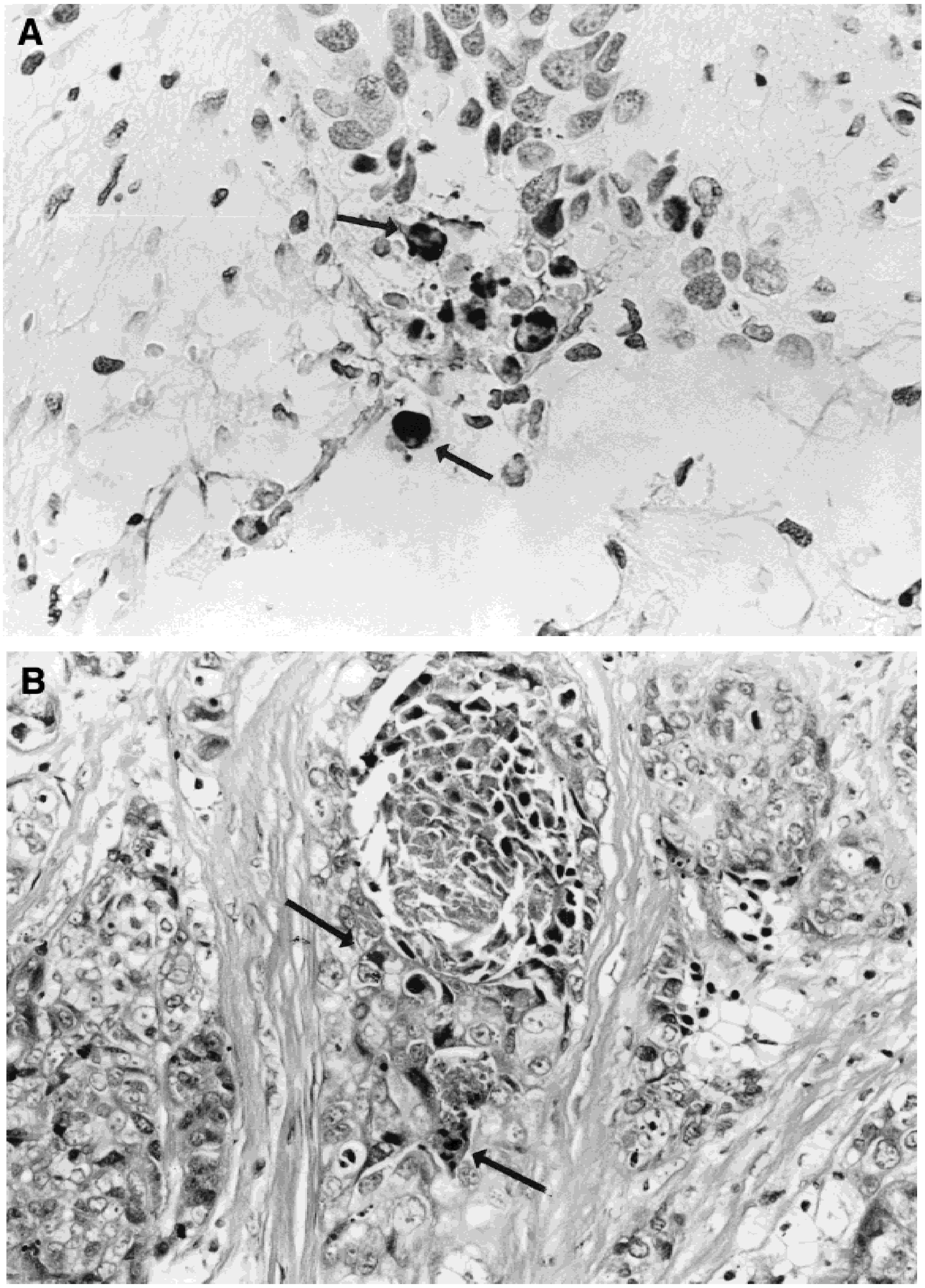

Fig. 1. High-grade breast carcinoma containing several apoptotic cells (arrows) in TUNEL staining (**A**: magnification ×500) and hematoxylin-eosin staining (**B**: magnification ×250). TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

Relationships Between Clinicopathological Factors and Mean Values of Apoptosis

Table I shows the relationships between clinicopathological factors and mean values (±SD) of apoptosis. There was no significant relationship between apoptosis and age, menopausal status, lymph node status, or f, while there were marginal relationships between apoptosis and ly or EIC. However, the mean value of apoptosis significantly increased with tumor size. Regarding histological type, mean values of solid-tubular carcinomas were significantly higher than those of papillotubular or scirrhous carcinomas. Tumors with overexpression of p-53 or higher DNA polymerase α values, and ER(−) tumors had higher apoptosis values than p53(−) tumors, tumors with lower DNA polymerase α values, or ER(+) tumors, respectively. Multivariate analysis revealed that factors independently correlated with apoptosis were p53, DNA polymeraseα, and tumor size.

TABLE II. Apoptosis and Cell Cycle by DNA Analysis/Proliferation in Breast Cancer*

Variables	No. of apoptotic cells 0–10	11–30	31+	*P* value
Cell cycle (%: mean ± SD)				
G_1	90.0 ± 10.9	85.7 ± 9.3	77.6 ± 10.1	<0.0001
S	4.7 ± 5.2	9.2 ± 6.7	15.1 ± 7.7	<0.0001
G_2+M	5.3 ± 7.7	5.1 ± 3.8	7.3 ± 5.0	0.94
No. of cases	47	35	24	106
Ploidy pattern (%)				
Diploid	32	13	8 (15.1)	53[a]
Aneuploid	15	22	16 (30.2)	53[a]
DNA polymerase α (%)				Total
0 to 19	42	25	5 (6.9)	72[b]
20 to 49	9	10	12 (38.7)	31[b]
50+	7	4	9 (45)	20[b]

*SD, standard deviation.
[a]$P = 0.004$.
[b]$P = 0.0002$.

Relationships Between Clinicopathological Factors and Apoptosis Classified Into 3 Categories

The average number of apoptotic cells in all cases was 19.9 per 5HPF (0–168) by H&E staining. To clarify the clinical significance of higher values or lower values of apoptosis, we divided the cases into 3 groups; 0 to 10, 11 to 30, or 31+ apoptotic cells per 5HPF. The distribution of cases according to the number of apoptotic cells was 70 cases with 0 to 10, 46 cases with 11 to 30, and 37 cases with 30+ apoptotic cells per 5HPF. Apoptosis correlated significantly with tumor size and histological type, as shown by the correlation of the mean value. There was also a significant relationship between apoptosis and p53 or ER. In addition, apoptosis was often seen in tumors with comedo component (Table III).

Relationship Between Apoptosis and Cell Cycle/Proliferative Activity

Table II shows the relationship between the proportion of cells in each phase of the cell cycle and apoptosis. Tumors with higher values of apoptosis had a significantly lower G1 phase and higher S phase fraction. Apoptosis correlated well with proliferative activity as assessed by DNA polymerase α. This indicates that apoptosis was more often observed in tumors in which cells were proceeding through the cell cycle. Furthermore, with regard to the ploidy pattern, tumors with higher levels of apoptosis were often seen in aneuploid cases.

Apoptosis and Prognosis

Regarding the relationship between prognosis and apoptosis, patients with higher values of apoptosis had a significantly poorer prognosis as indicated by overall and cumulative disease-free survival, as shown in Figure 2. Table III shows that postoperative therapy was not biased in any apoptosis group.

Uni- and Multivariate Analysis of Clinicopathological Factors for Overall Survival in Breast Cancer

Table IV shows uni- and multivariate analyses of clinico-pathological factors for overall survival in breast cancer. In univariate analysis, apoptosis, nodal status, DNA polymerase α, ly, P53, and ER status were significant factors. Among these factors, ER status was the only significant factor in multivariate analysis and the following independent factor was DNA polymerase α. Although apoptosis was not a significant factor in multivariate analysis, apoptosis might be a useful predictor for prognosis as it significantly correlated with ER and DNA polymerase α.

DISCUSSION

Recent reports have showed that both radiation and anticancer agents induced cell death in the treatment of cancer [9,10,21], although there were differences depending on the target cells and mechanisms of action of different types of therapy. It has also been shown that this apoptosis occurs even in cases with ovariectomy [13], and also in cases with TAM (tamoxifen) [11,12] as endocrine therapy in breast cancer. It is thought that the tumor suppressor gene p53 guides a cell toward apoptosis when it is judged as impossible to repair and when the cell cycle is arrested at G1 phase after trauma or when abnormality of genes is recognized [22,23]. It is conceivable that this mechanism suppresses cancerization by guiding a cell to apoptosis. In reports that have evaluated the function of apoptosis in breast cancer, bcl-2 expression was closely related to positive ER and maintaining a long life by avoiding apoptosis [24,25]. This suggests that patients with lower apoptosis have a favorable prog-

TABLE III. Apoptosis (Classification to 3 groups) and Clinicopathological Variables in Breast Cancer*

Variables	Apoptotic cells 0–10	11–30	31+	Total	*P* value
Age (years)					
−35	2	1	1 (25.0)	4	
36–50	32	26	12 (17.1)	70	0.80
51+	36	19	14 (20.3)	69	
Menopausal status					
Pre	35	27	14 (18.4)	76	0.65
Post	35	19	13 (19.4)	67	
Tumor size (mm)					
−10	20	16	1 (2.7)	37	
11–20	42	26	14 (16.7)	84	0.002
21+	8	4	10 (45.5)	22	
Nodal status					
−	52	38	16 (9.4)	106	0.09
+	18	8	11 (29.7)	37	
Histological type					
Non-invasive carcinoma	3	2	0	5	
Papillotubular carcinoma	13	6	3 (13.6)	22	0.09
Solid-tubular carcinoma	12	16	11 (28.2)	39	
Scirrhous carcinoma	34	21	11 (16.7)	66	0.10
Lobular carcinoma	2	1	0	3	
Mucinous carcinoma	4	0	0	4	
Others	2	0	2	4	
ly					
−	34	20	7 (11.5)	61	
+	25	18	13 (23.2)	56	0.37
++	11	8	7 (26.9)	26	
f					
−	21	15	8 (18.6)	45	
+	35	22	11 (16.2)	68	0.96
++	14	10	7 (22.5)	31	
EIC					
−	47	33	20 (20.0)	100	0.76
+	23	13	7 (16.3)	43	
Comedo					
−	18	9	2 (6.9)	29	0.048
			5 (35.7)	14	
+	5	4			
p53					
−	44	18	6 (8.8)	68	
+	21	17	3 (7.3)	41	<0.0001
++	5	11	18 (52.9)	34	
ER					
+	44	29	5 (6.4)	78	<0.0001
−	17	11	22 (44.0)	50	
Unknown	9	6	0	15	
Adjuvant therapy					
None	9	7	3 (15.8)	19	
Chemotherapy	22	14	10 (21.7)	46	0.16
TAM	17	8	0	35	
Chemotherapy + TAM	22	17	14 (27.5)	51	

*ly, lymphatic invasion; f, infiltration to fatty tissue; EIC, extensive intraductal component; ER, estrogen receptor; TAM, tamoxifen.

nosis. It was demonstrated in the present study that overexpression of p53 significantly correlated with a higher value of apoptosis. As the half-life of wild-type p53 protein is short and that of mutant-type p53 protein is long, most p53 protein detected immuno-histochemically may be that of the mutant type, which has a tendency to be accumulated in the nucleus [26,27]. Many investigators [28,30]havereported that overexpression of p53 in breast cancer is related to aggressive characteristics such as ER (-), a high degree of histological grade, or higher proliferative activity.

We demonstrated that apoptosis was more often seen

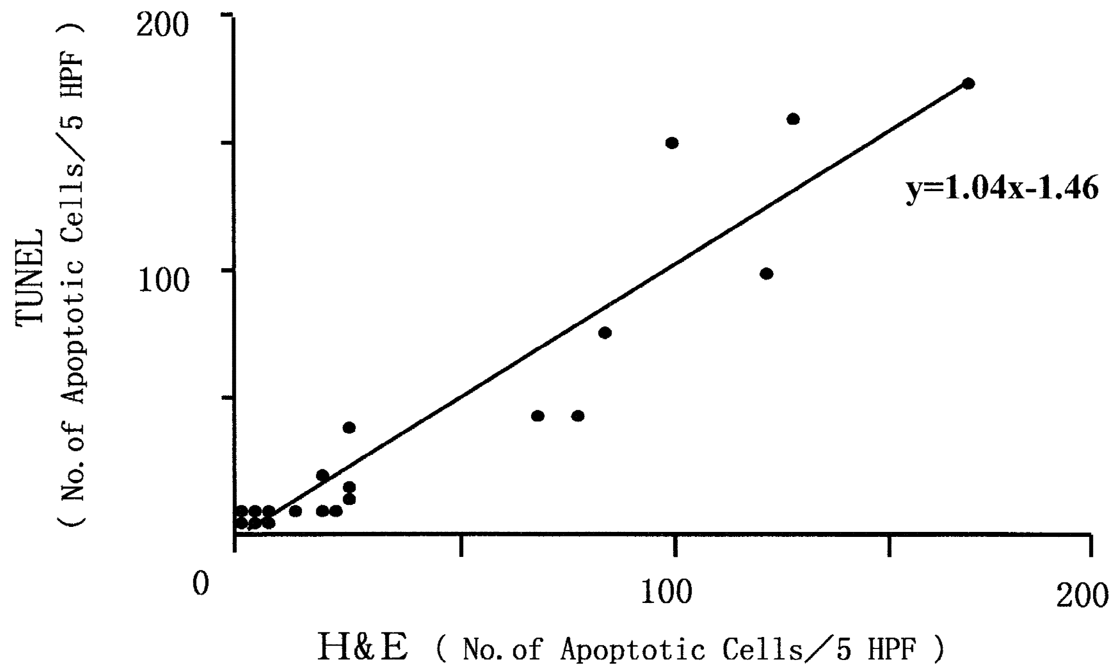

Fig. 2. Comparison of numbers of apoptotic cells estimated by hematoxylin-eosin staining versus TUNEL staining in 20 breast cancer tumors. There was a significant correlation between the two types of staining ($P < 0.001$). TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

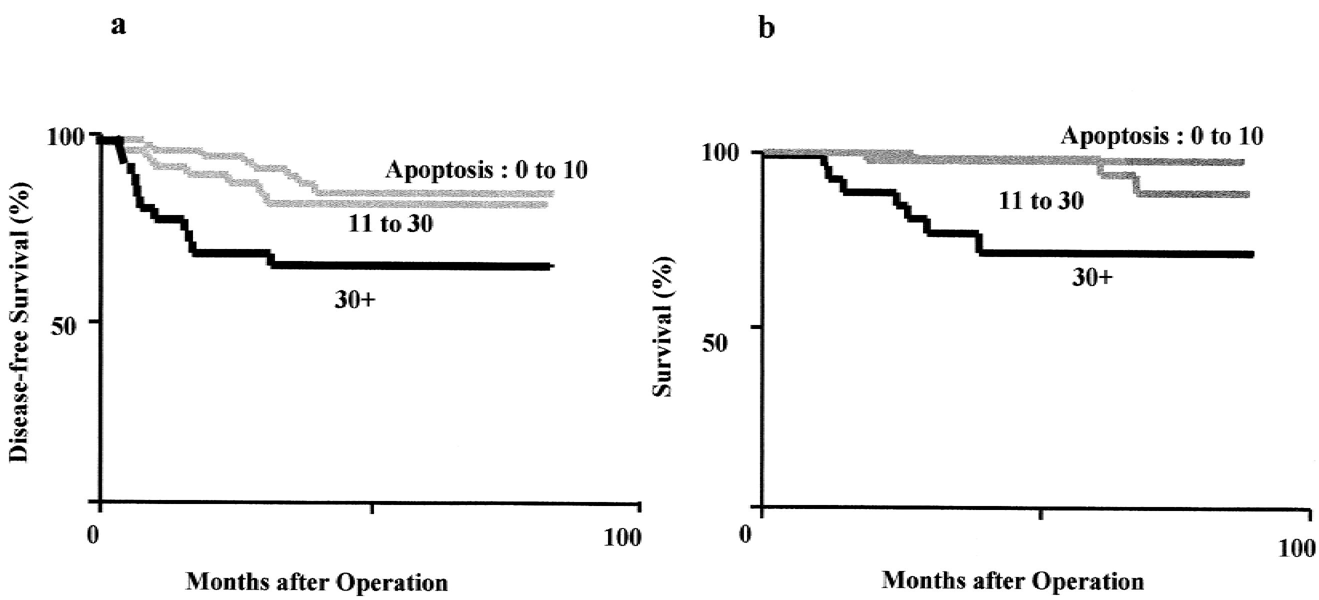

Fig. 3. Disease-free (**a**) and overall survival (**b**) according to apoptosis (No./5 HPF) in breast cancer. Patients with higher values of apoptosis had a significantly poorer prognosis ($P = 0.007$ and $P = 0.0001$, respectively). HPF, high-power field.

in larger tumors, ER(-) tumors, aneuploid tumors, tumors with lower G1 fraction and higher SPF as shown by cell cycle analysis, and tumors with higher values of DNA polymerase α. These findings indicate that apoptosis is correlated with the degree of biological aggressiveness and proliferative activity, and that cases with higher values of apoptosis had poorer prognosis. Regarding this point, Lipponen et al. [31] reported that apoptosis correlated with histological malignancy, degree of atypia, higher SPF, ER (−), and overexpression of p53, and that cases with more apoptosis had poorer prognosis. Bodis et al. [32] reported that there was a significant relationship between apoptosis and high-grade DCIS (ductal carcinoma in situ), and that cases with more apoptosis had an unfavorable prognosis irrespective of p53. Regarding the relationship with proliferative activity, Komada et al. [33] reported the induction of apoptosis through the Fas pathway via promoting progression through the cell

TABLE IV. Uni- and Multivariate Analysis of Clinicopathological Factors for Survival in Breast Cancer*

Factors	Category	Relative risk	*P* value (univariate)	Wald Chi-square	*P* value (multivariate)
Apoptosis	11–30/0–10	3.45	0.07	5.55	0.57
(number/5HPF)	31+/0–10	5.48	0.02		
Age (years)	36–50/−35	0.66	0.70	1.11	
	51+/−35	0.44	0.46		
Lymph node status	+/−	4.99	0.008	7.12	0.26
Tumor size (mm)	11–20/−10	3.69	0.21	4.27	
	21+/−10	5.48	0.13		
DNA polymerase α (%)	20+/−19	9.30	0.004	8.38	0.07
Lymphatic invasion	+/−	3.37	0.05	3.81	0.35
p53	+/−	0.62	0.56	5.98	0.45
	++/−	3.63	0.02		
Ploidy pattern	Aneuploid/diploid	1.65	0.35	0.86	
Estrogen receptor	+/−	0.14	0.003	8.53	0.008

*HPF: high power field.

cycle of leukemic cells. Samoszuk et al. [34] reported that an elevated apoptotic rate had an association with high SPF. Thus, ER (−) tumors and tumors with higher proliferative activity showed higher values of apoptosis in accordance with the reports that bcl-2 expression was closely correlated to ER (+) and avoidance of apoptosis. From this, we conclude that increased apoptosis indicates poor prognosis.

Apoptosis is often seen in tumors with comedo type, although no obvious relationship between the mean values of apoptosis and EIC-comedo status has been observed. It is important, especially in breast-conserving surgery, to compare comedo with non-comedo type, and we have reported [35] that cases with comedo type might have a poorer prognosis than those with non-comedo type. Kobayashi et al. [36] reported that the papillary-cribriform type intraductal components expressed both bcl-2 and ER proteins more often than the solid-comedo type. Necrosis is characteristic of comedo cases, and there was a significant relationship between necrosis and apoptosis in this study. It is generally thought that proliferation/regression of a tumor is dependent on the balance of cell gain (proliferation) and cell loss (apoptosis and necrosis). Tumor regression occurs only when the rate of tumor cell death becomes greater than that of tumor cell proliferation. Tumors with higher values of apoptosis, however, have not only more necrosis but also higher proliferative activity.

In conclusion, apoptosis in breast cancer reflects biological behavior, and tumors with a higher value of apoptosis possess a higher degree of biological aggressiveness and proliferative activity and indicate a poorer prognosis. Two of the most important and interesting problems are what type of treatment should be recommended for rapidly growing tumors with more apoptosis vs. slowly growing tumors with less apoptosis, and the nature of the effects of the therapy.

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